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GRANT NUMBER DAMD17-96-1-6301

TITLE: Novel Approaches to Preventing Urinary Tract Infection in Women

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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19980116 169

DTIC QUALITY INSPECTED 3

REPORT DOCUMENTATION PAGE

Form Approved

OMB No. 0704-0188

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|---|---|--|---------------------------------------|---|--|
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE September 1997 | | 3. REPORT TYPE AND DATES COVERED Annual (1 Sep 96 - 31 Aug 97) | |
| 4. TITLE AND SUBTITLE Novel Approaches to Preventing Urinary Tract Infection in Women | | | | 5. FUNDING NUMBERS DAMD17-96-1-6301 | |
| 6. AUTHOR(S) Ann E. Stapleton, M.D. | | | | | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington Seattle, WA 98105-6613 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012 | | | | 10. SPONSORING/MONITORING AGENCY REPORT NUMBER | |
| 11. SUPPLEMENTARY NOTES | | | | | |
| 12a. DISTRIBUTION / AVAILABILITY STATEMENT DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Sep 97). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012. | | | | 12b. DISTRIBUTION CODE | |
| 13. ABSTRACT (Maximum 200) Urinary tract infections (UTIs), generally caused by <u>Escherichia coli</u> or <u>Staphylococcus saprophyticus</u> , are extremely common among young women and 25% of these patients develop frequent recurrent infections. Although UTIs can be treated, we currently lack effective means to prevent UTI in women. A necessary prerequisite to UTI is adherence of uropathogens to the vaginal and bladder epithelium. Preliminary data for this project showed that as in the kidney, specific host cell glycosphingolipids (GSLs) on the vaginal and bladder epithelium appear to function as receptors for uropathogenic <u>E. coli</u> and <u>S. saprophyticus</u> . This report describes the first year of progress in a project whose overall goal is to define uropathogen-binding GSLs in the vaginal and bladder epithelium as a prerequisite to the rational design of new agents that will prevent colonization and infection in women. Key progress includes: (1) Demonstrating the feasibility of using primary epithelial cell cultures for GSL identification and purification; (2) Establishment of primary cultures of vaginal epithelial cells; (3) Definitive demonstration of globoseries GSLs in extracts of GSLs from primary cultures of bladder and vaginal epithelial cells, enriched as compared with continuous malignant cell lines; and (4) The first unambiguous structural determination of SGG isolated from a normal human tissue. | | | | | |
| 14. SUBJECT TERMS Urinary Tract Infection, <u>E. coli</u> Defense Women's Health Research Program | | | | 15. NUMBER OF PAGES 28 | |
| | | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT Limited | | |

FOREWORD

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Anne Stapleton MD 9/28/97
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5. INTRODUCTION

a. Overview

The overall purpose of this project is to investigate interactions between bacteria which commonly cause urinary tract infection (UTI) and their cognate host cell receptors in the vaginal and bladder epithelium in order to design novel, non-antibiotic methods for preventing UTIs. The project is focused on studying the two most common uropathogens causing UTI in young women, Escherichia coli and Staphylococcus saprophyticus, as well as their interactions with glycosphingolipids (GSLs) on the cell surface of the bladder and vagina. In the first two years of this project, we will define the key GSLs on the eukaryotic cell surface that uropathogenic bacteria use for attachment and then in the last two years, we will take advantage of new biochemical techniques using carbohydrate mimetics to design UTI prevention methods that avoid the induction of antimicrobial resistance. This report describes progress made in the first of four years of this grant.

b. Background

Acute uncomplicated UTIs caused by E. coli and S. saprophyticus occur in an estimated 7 million young women each year at an annual cost for diagnosis and treatment exceeding one billion dollars. Over half of all women have had a bacterial UTI by their late 20's and approximately 20% of women with UTI suffer very frequent ($\geq 3/\text{year}$) recurrences (1). Nonetheless, the only currently available preventive modality for these recurrent infections is antimicrobial prophylaxis. Though effective, antimicrobial prophylaxis may promote the emergence of drug-resistant strains (2). In addition, women typically revert to having frequent recurrences once prophylaxis ceases and little is known about why some women suffer frequent recurrences of UTI, since this phenomenon cannot usually be explained by underlying functional or anatomic abnormalities of the urinary tract (2). The interaction of infecting bacterial strains with the women's epithelial cells appears to be a critical point in the infectious process that determines host susceptibility, in particular the availability and nature of host cell bacterial ligands such as GSLs (3, 4).

GSLs are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, to which organisms attach, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane. They serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including E. coli, Pseudomonas aeruginosa, Helicobacter pylori, HIV, parvovirus, rotavirus, cholera toxin, verotoxin of E. coli 0157, and others (5-9). Based on the structures of their carbohydrate components, they are grouped into families, such as the lacto- and neolacto-series, the globoseries, and the ganglioseries GSLs. GSLs are synthesized by the sequential action of glycosyltransferases, many of which are tissue-specific and/or genetically determined (10). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases (5).

Among uropathogenic E. coli, isolates expressing the pap -encoded family of adhesins are significantly overrepresented among strains collected from patients with UTI as compared with fecal isolates from patients without UTI (11). The GSL receptors for these adhesins in the kidney are the globoseries GSL family that contain a minimal receptor consisting of a galactose α -1-4 galactose moiety (11). Although S. saprophyticus is the second most common cause of UTI, to our knowledge, we are the first to actually investigate whether it binds to GSLs. In our grant proposal, we showed preliminary data demonstrating that the

wild-type S. saprophyticus strain ST352 binds asialo GM1 (ASGM1), a neutral ganglioseries GSL and that other wild-type S. saprophyticus isolates bind to ASGM1 and/or structurally-related ganglioseries GSLs. Paradoxically, while globoseries GSLs have been identified in kidney tissue and vaginal epithelium, the bladder has been little studied with respect to GSLs, despite the fact that it is the most common site of UTI. Previous studies of GSLs in native bladder tissues have focused on oncogenesis or development and have not included GSLs that are directly involved in adhesion of uropathogens (ganglioseries and globoseries GSLs). Of note, other urogenital pathogens, including C. albicans, C. trachomatis, N. gonorrhoeae, have also been reported to bind to ASGM-1 and it is possible that a single class of inhibitors could prevent adherence and infection with all of these agents.

c. Brief summary of preliminary data presented in original proposal

In the original proposal, we presented preliminary data demonstrating that primary cultures of human bladder epithelial cells appear to be an promising model system for the study of bladder GSLs in the pathogenesis of UTI caused by E. coli or S. saprophyticus. Specifically, we showed that these cell cultures appear to express globoseries GSLs, the host cell binding ligand for an important class of uropathogenic E. coli, those expressing pap-encoded adhesins. We and others have previously shown that SGG and other globoseries GSLs are surface exposed in human kidney tissues and exfoliated vaginal epithelial cells (3, 12). In our preliminary immunocytology experiments with primary cultures of human uroepithelium, the cells demonstrated bright immunofluorescent staining with MAb ID4 directed against SGG, suggested that this epitope is also surface exposed on these bladder cells. We also showed that S. saprophyticus, the second most common cause of UTI in young women, binds to ganglioseries GSLs, especially ASGM1 and ASGM2. In addition, we demonstrated the presence of ASGM1 among GSLs extracted from human kidney and the surface exposure of this epitope in kidney sections in specific histological areas where bacteria also adhere. Another ganglioseries GSL, GM1, was identified among GSLs extracted from human kidney tissues and vaginal epithelial cells. ASGM1 appears to be surface exposed on both kidney and cultured primary bladder cell surfaces, as shown by positive immunofluorescent staining with MAb TKH-7, directed against ASGM1.

d. Originally proposed hypotheses

The original hypotheses of this project have been supported by data obtained during the first year of the grant and thus remain unchanged, as listed below. The overall goal of this project remains to define the key eukaryotic cell surface GSLs that are used by uropathogenic bacteria for attachment and then to take advantage of new biochemical techniques utilizing carbohydrate mimetics to design novel means for preventing UTIs that avoid the use of antimicrobials.

- (1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for E. coli and S. saprophyticus, respectively.
- (2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.
- (3) We hypothesize that carbohydrate mimetic and synthesis techniques can be used to design high-affinity inhibitors of E. coli and S. saprophyticus binding to vaginal and bladder transitional epithelium.

e. Original technical objectives

Our original technical objectives remain unchanged at this point in the project. Because of some unique collaborative opportunities that developed during the past year, we have accomplished some tasks originally delegated to later years of funding. We have also experimented with changing a technical aspect of one of the tasks planned for the first year and have thus postponed completing this task until year 2. These changes are discussed in greater detail below. Our technical objectives are as follows:

(1) We will extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional epithelium and vaginal epithelium, according to the following sequence: (a) purify the GSLs using high-performance liquid chromatography (HPLC); (b) identify bacteria-binding GSLs by overlaying radiolabeled isolates of E. coli and S. saprophyticus on these GSLs separated on high-performance thin-layer chromatography (HPTLC); (c) confirm the identities of these GSLs using specific monoclonal antibodies (MAbs) directed against the GSLs in HPTLC immunostaining assays; and (d) perform carbohydrate structural analysis on the bacteria-binding GSLs.

(2) To demonstrate that the GSLs identified in Hypothesis 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection, we will: (a) test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures; (b) utilize immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs; (c) repeat 2a and 2b after pretreatment of the cell cultures with an inhibitor of GSL receptor synthesis; and (d) repeat 2a after pretreatment of the cell cultures with the MAbs directed against relevant GSLs.

(3) We will use carbohydrate mimetic techniques to design inhibitors of bacterial adherence, focusing initially on the interaction of E. coli with sialosyl galactosyl globoside (SGG, a GSL to which E. coli binds with high affinity; see preliminary data). We will test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays, as described in the second objective.

6. BODY OF REPORT

A. Overview

During the first year of funding, our investigations have largely followed the original plan of work in the same order of tasks shown below. However, changes occurred in the affiliations of some of the personnel involved in the study and unique collaborative opportunities occurred, resulting in our being able to begin some of the tasks described in Technical Objective 2. The changes and opportunities we encountered this past year included:

(1) The dissolution of the Biomembrane Institute. The Biomembrane Institute was dissolved as a non-profit entity and Dr. Hakomori's laboratory remained unchanged but moved to the Pacific Northwest Research Foundation in Seattle. Dr. Hakomori's role and commitment as an unpaid volunteer consultant remain unchanged and he and his group continue to provide reagents and intellectual assistance to this project. As an example, a member of Dr. Hakomori's group whose own project included development of inhibitors of carbohydrate

synthesis learned of our interest in such compounds relevant to Task 3 of Technical Objective 2. He then assisted us in applying this technology to our primary cell culture system, enabling us to successfully perform experiments shown below that demonstrate the critical importance of GSLs in mediating the adherence of uropathogenic *E. coli* expressing *pap*-encoded adhesins to bladder epithelial cells.

(2) Movement of Dr. Stroud to the Molecular Medicine Department at Northwest Hospital in Seattle. Dr. Stroud is now part of a oncology-related carbohydrate research group located in Northwest Hospital and headed by Dr. Eric Holmes, an expert in glycosyltransferases involved in the synthesis of GSLs. Dr. Holmes has demonstrated a strong commitment to supporting Dr. Stroud's work as a co-investigator on this project. He has provided Dr. Stroud with space for his own work on this project as well as allowing full access to the facilities for Amy Denton, the Research Technician hired to assist on this project. As a result, the project has continued as planned in the original proposal, with respect to Dr. Stroud's role. The minor change that has resulted from Dr. Stroud's move is our effort to develop a non-radioactive method for immunostaining thin layer chromatography (TLC) plates, described below. Although facilities for labeling and using $^{125}\text{-I}$ protein A are available at Northwest Hospital and the University of Washington, Dr. Stroud had already begun developing a method for immunostaining without the use of $^{125}\text{-I}$ protein A. Thus, we elected to try to develop such a method further before turning to an alternative, such as arranging to use a core facility for radioactive iodine at the University of Washington. The preliminary results of these efforts are described below.

(3) Establishment of a new collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens. Dr. Levery is the Co-Technical Director of the Resource Center for Biomedical Complex Carbohydrates at the CCRC and an eminent structural biochemist in the field of GSLs. He is responsible for the structural analysis of diverse glycoconjugates using proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$), mass spectrometry (MS), and gas chromatography-mass spectrometry (GC-MS). The CCRC is equipped with two high-field NMR spectrometers (Bruker AM-500 and AMX-600), with the purchase of an additional instrument in the 750-800 MHz range planned for the near future. In addition, the Center has numerous and diverse mass spectrometric instruments, including laser-desorption time-of-flight (Linear Scientific LDI-1700), electrospray triple-quadrupole (Sciex API-III), and high-resolution four-sector (JEOL JMS-SX/SX102A) mass spectrometers, as well as various instruments for LC, GC, and GC-MS. All of these are dedicated to carbohydrate and glycoconjugate research. Drs. Stapleton, Stroud and Hakomori have previously collaborated with Dr. Levery in a very productive fashion. Now in collaboration with Dr. Levery, we have recently accomplished the first unambiguous structural characterization of sialosyl galactosyl globoside (SGG) from normal human tissue, as described below. A copy of the standard National Institutes of Health Biosketch provided to us by Dr. Levery is included in the appendix.

(4) Establishment of a collaboration with Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchison Cancer Research Institute and the University of Washington. Drs. McElrath and Hladik have established a system for the short-term culture of normal vaginal and cervical tissues obtained at surgery. These studies, ongoing for more than a year, have been fully approved by the Human Subjects Division at the University of Washington and their purpose is to obtain genital tissue lymphocytes for studies unrelated to ours. The epithelial portions of these tissue cultures were previously being discarded but are now provided to us at intervals. Using these tissues, we have been able to establish pure primary vaginal epithelial cell cultures and to extract GSLs therefrom, as described below.

B. Original Statement of Work

The original technical objectives set for the first two years of funding are listed below. Tasks on which we have made progress are noted in bold italic.

Technical Objective 1: Extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional and vaginal epithelium.

- Task 1:** *Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells*
- Task 2:** *Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures*
- Task 3:** *Months 7 to 12: bacterial overlay assays*
- Task 4:** *Months 7 to 12: immunostaining assays*
- Task 5:** *Months 13 to 24: carbohydrate structural analysis*
- Task 6:** Months 25 to 36: data analysis and publication

Technical Objective 2: Demonstrate that the GSLs identified in Technical Objective 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

- Task 1:** *Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures*
- Task 2:** Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs
- Task 3:** *Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays*
- Task 4:** Months 24 to 36: MAb pre-treatment, followed by GSL extraction and quantification and bacterial adherence assays
- Task 5:** Months 30 to 36: data analysis and publication

C. Details of Progress

1. Technical Objective 1

a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala and maintained in serum free keratinocyte media using standard tissue culture techniques, as in our preliminary studies and as he has described (13). Briefly, cells were maintained in serum- and antibiotic-free keratinocyte medium and passed at 70% confluence in a ratio of 1:4 or 1:6 (13). At each passage, the maximal degree of expansion was achieved with the

goal of moving on to Task 2 as quickly as possible, in order to assess how many flasks of cells are ultimately needed over the course of this project to purify GSLs of interest. This procedure was continued until senescence was noted, usually at about passage 12.

In order to confirm the need for using primary bladder epithelial cells, we also cultivated two standard continuous, malignant bladder cell lines obtained from ATCC, namely T24 cells and J82 cells. Our prediction from the literature was that the globoseries GSLs of these cells should be altered because of their malignant origin, but these GSLs have been relatively little studied. Thus, we chose to perform a limited series of experiments to culture and harvest these cells for a characterization of their GSL content with respect to those moieties capable of binding a pap-encoded adhesin expressing E. coli isolated in a bacterial overlay assay. These malignant cell lines have been also used by other investigators to study adherence of pap-encoded adhesin expressing uropathogenic E. coli (14). These were maintained per the ATCC protocol provided with the cells. Cells were grown in Minimal Essential Medium (MEM) with 10% fetal calf serum and flasks were passed at 90 % confluence. Again, cells were maximally expanded and harvested until a pellet of 1 mL was obtained for GSL analysis.

Primary cultures of vaginal epithelial cells were established de novo in our laboratory through the collaboration with Drs. McElrath and Hladik, as described above. After a thorough exploration of the scant literature on this subject, we tried several methods of cultivating these cells. Biopsy specimens were placed in cold MEM with d-valine (d-val), penicillin-streptomycin (P-S), and insulin-transferrin-selenium (ITS) and kept on ice until processing. Because of overgrowth of fibroblasts during early attempts in which less microdissection was attempted, biopsies were then microdissected to remove stroma. They were twice put through a procedure of rinsing quickly with ethanol, cutting the tissue into small pieces, placing it in sterile PBS and centrifuged briefly. The supernatant was aspirated and collagenase was added with MEM d-val with P-S and ITS. The suspension was placed to shake at 4° C overnight or at 37°C for 2 hours in CO₂ and then centrifuged again, resuspended in media without antibiotics and divided into flasks. When the cells had been maintained for approximately ten days without the appearance of fibroblasts, the media was changed to the same keratinocyte medium used for the bladder epithelial cells or MEM d-val ITS. Again, cells were grown until senescence was observed, usually about 5 to 6 passages. Cell lines with fibroblast contamination were discarded.

2. Results and discussion

Primary bladder epithelial cells: For this series of experiments, 4 mL of cells (225 cm² flasks) were harvested as pellets and saved for GSL extraction as described below. 250 flasks of cells were cultivated over 4 months to accomplish this, not including those flasks maintained to assure continuous passage.

J82 and T24 bladder cell lines: For this series of experiments, a 1 mL packed cell pellet of each cell line was harvested and saved for GSL extraction as described below.

Primary vaginal epithelial cells: A total cell pellet of 9 mL was obtained for use in the GSL extraction experiments described below. This included pooled samples from pilot experiments which likely contained some stromal material, as they preceded the improvement of the microdissection technique. Additional harvesting of pellets is ongoing at this writing.

3. Recommendations in relation to the Statement of Work

1. Primary bladder epithelial cells: We plan to continue to cultivate bladder cell lines as needed for further purification of GSLs throughout the next year of funding and likely beyond.
2. J82 and T24 bladder cell lines: We will no longer use these cell lines, having demonstrated their unsuitability for these projects. We will include the data regarding expression of globoseries GSLs in these cells in a manuscript to be prepared on the primary cell model of urinary tract infection.
3. Primary vaginal epithelial cells: In the next funding period, we will continue to share tissue samples with Drs. McElrath and Hladik and use them in the establishment of new primary vaginal epithelial cell lines. Although these activities are already IRB approved, we have filed a certificate of exemption for the secondary use of these tissues. We will also obtain primary vaginal epithelial cell lines established by Dr. Atala to compare their adherence and GSL characteristics with those of our cell lines. In addition, we will try other changes in our procedures to propagate the cells longer, such as the use of estrogen and/or progesterone. There is a paucity of data in the literature on this subject and this work will be groundbreaking in itself, as are our efforts to date. Thus, we will prepare a publication describing the results of the various methods we have employed to maximize the culture of these cells.

b. Task 2, Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures

1. Experimental methods, assumptions and procedure

Extraction of GSLs from primary cultures of human bladder epithelial cells and vaginal epithelial cells was performed as in our preliminary data and our previous work using exfoliated vaginal epithelial cells (3). At each step, GSLs were chromatographed in various organic solvent systems (15) and stained with orcinol (carbohydrate detection stain) to assess the purity of individual bands. At each step, HPTLC bacterial overlay procedures as described below were performed to identify and monitor the purification of GSLs of interest. Briefly, the GSL isolation and purification steps are as follows: cell cultures were maintained as described (13) then bladder cells were trypsinized, pelleted, and washed, and the total GSLs were obtained by extracting the pellets with 10 volumes isopropanol:hexane:water (IHW; 55:25:20 by volume) with sonication in a warm bath and centrifugation at 2,500 RPM for 10 minutes. To obtain the upper and lower phase GSLs, the supernatant was then dried under nitrogen and twice phase partitioned using the Folch procedure (16). To separate neutral GSLs from gangliosides, we subjected the upper phase GSLs to reverse phase column chromatography, followed by anion exchange chromatography (17). Further purification of GSLs that bind E. coli or S. saprophyticus is presently in progress, performed by preparative HPTLC (18) and/or high-performance liquid chromatography (19).

2. Results and discussion

Examples of the purification steps described above as applied to the vaginal epithelial cell samples are shown in Figures 1 and 2 (Folch partitioning and reverse phase column chromatography). Yields from these studies were approximately as expected, except that the overall yield from primary vaginal epithelial cells is less than that of primary bladder epithelial cells because the cells appear not to be able to survive as long yet in culture. Examples of the purification of primary bladder epithelial cells and of continuous malignant bladder cell lines are shown in later figures related to Task 3.

1 2 3 4 5 6 7

Figure 1. Orcinol stained thin-layer chromatography plate

Orcinol stained TLC plate showing example of purification steps used for bladder and vaginal epithelial cell GSL extractions: total organic extractions after Folch partitioning. GSLs extracted from cervical epithelial tissues for another project (performed by personnel not funded by this project) are included as a control.

Lane 1: cervical epithelial lower phase; **Lane 2:** cervical upper phase; **Lane 3:** vaginal epithelial cell lower phase; **Lane 4:** blank; **Lane 5:** vaginal epithelial cell lower phase; **Lane 6:** standard asialo GM2; **Lane 7:** standard ceramide trihexosyl (CTH).

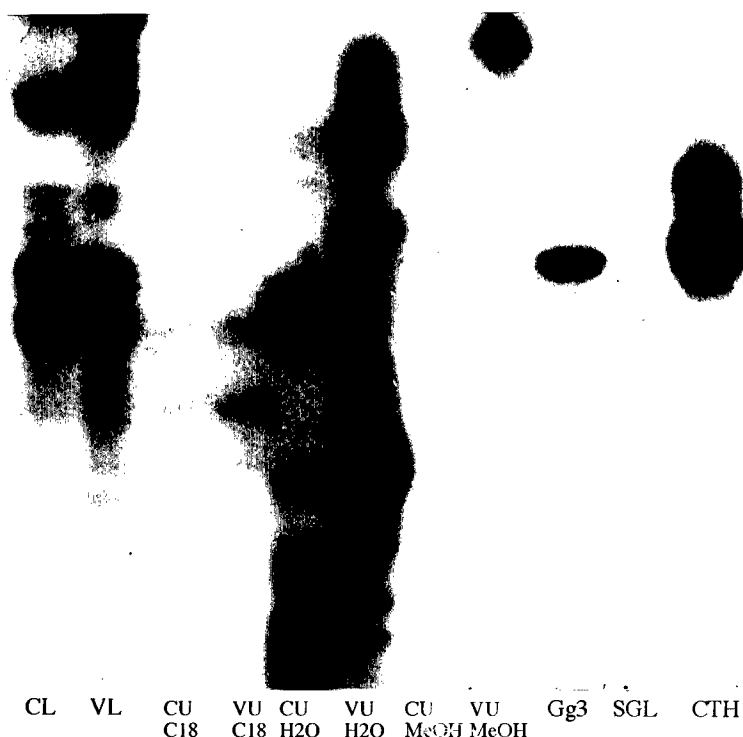


Figure 2. Orcinol stained thin-layer chromatography plate

Orcinol stained TLC plate showing example of purification steps used for bladder and vaginal epithelial cell GSL extractions: fractions after reversed phase column chromatography. GSLs extracted from cervical epithelial tissues for another project (performed by personnel not funded by this project) are included as a control. V, vaginal; U, upper phase, L, lower phase; MeOH: methanol; Gg3, standard asialo GM2; SGL, standard sialosyl galactosyl globoside; CTH, standard ceramide trihexosyl.

Lane 1: cervical lower phase; **Lane 2:** vaginal lower phase; **Lanes 3 and 4:** cervical and vaginal upper phases, respectively, after 100% methanol wash; **Lanes 5 and 6:** cervical and vaginal upper phases, respectively, after water wash; **Lanes 7 and 8:** cervical and vaginal upper phases, respectively, after 50% methanol wash; **Lane 9:** standard asialo GM2; **Lane 10:** standard SGG; **Lane 11:** standard CTH.

3. Recommendations in relation to the Statement of Work

1. We confirmed our underlying assumption that it is necessary to use normal primary bladder epithelial cells rather than continuous malignant bladder cell lines in order to study the GSLs of interest in uropathogenesis (see Task 3 data). We have also gathered data on globoseries GSL expression in T24 and J82 cells that will be of interest to glycobiologists and tumor biologists.
2. As described above, we will continue with efforts to perpetuate the primary vaginal epithelial cells longer periods of time.
3. We have purchased a rotoevaporator, basic equipment for GSL drying, for the University of Washington laboratory to augment the instrumentation available through Dr. Stroud. This will

improve efficiencies, since it would be helpful to simultaneously carry out complementary purification steps in the two laboratories.

c. Task 3, Months 7 to 12: bacterial overlay assays

1. Experimental methods, assumptions and procedure

This assay involves separating GSLs on HPTLC plates and overlaying the plates with radiolabeled bacteria (3, 20). E. coli was metabolically labeled with [³⁵S]methionine as previously described (3). Briefly, organisms grown overnight on Luria or blood agar were scraped and resuspended in M9 medium, shaken for 40 minutes, then 200 µCi of [³⁵S]methionine was added. The organisms were shaken for an additional hour, washed, and resuspended in PBS. S. saprophyticus were grown overnight shaking in trypticase soy broth and metabolically labeled with [³⁵S]methionine using a gonococcal labeling method (Mandrell, unpublished data) that we adapted for S. saprophyticus. The organisms were incubated in RPMI 1640 medium without methionine (Gibco) for one hour, with the addition of [³⁵S]methionine. During this incubation, the organisms grow minimally but metabolize and incorporate [³⁵S]methionine, with a final specific activity of 0.01 cpm/organism, similar to the specific activity achieved using comparable methods for E. coli (3). Before use in the bacterial overlay assay, organisms were washed twice in PBS and aliquots were removed for quantitation of radioactivity and measurement of optical density.

For chromatography, 5-10 µg/lane of each GSL sample was spotted on glass HPTLC plates (Whatman) and the plates were chromatographed in chloroform:methanol:water (50:40:10) with 0.05 % CaCl₂, with one plate run in parallel for orcinol staining. Plates were then dried, dipped for 2 minutes in diethyl ether containing 0.5% polyisobutylmethacrylate, dried, and preincubated in bovine serum albumin (BSA)/PBS for one hour, then washed three times in PBS. Radiolabeled bacteria were then overlaid (10⁸ cpm total per plate) and the plates are gently rocked for one hour, washed four times in PBS and subjected to autoradiography.

In the first year of funding, for the bacterial overlay assays with E. coli, we have primarily used metabolically labeled wild type E. coli R45 (3), which has the pap class II adhesin genotype and phenotype and thus specifically recognizes globoseries GSLs.

2. Results and discussion

Examples of bacterial overlay assays performed to assess the identities of bacterial-binding GSLs among the compounds isolated from primary bladder epithelial cells and from primary vaginal epithelial cells are shown in Figures 3 and 4 below.

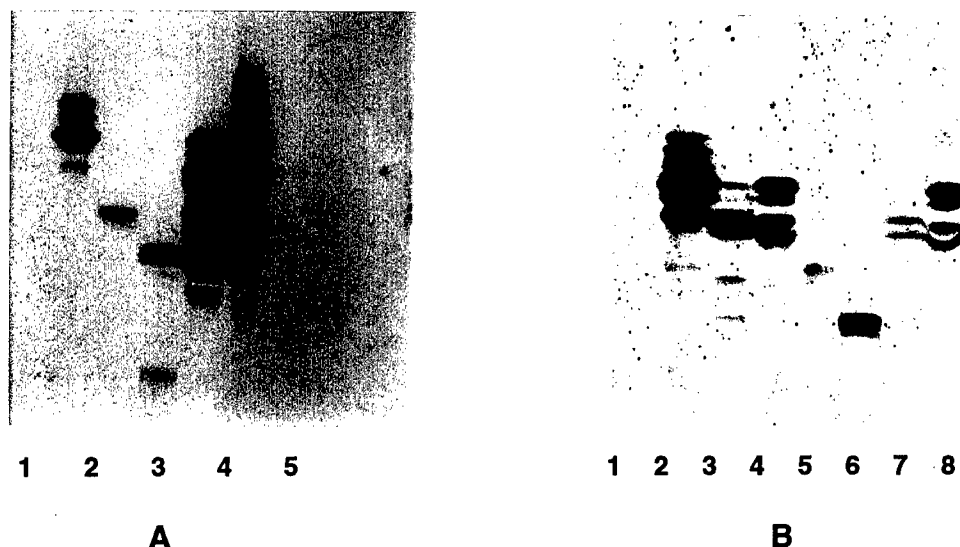


Figure 3. Binding of *E. coli* R45 to glycosphingolipids purified from human primary bladder epithelial cultures and from continuous malignant bladder cells lines T24 and J82

Primary bladder epithelial cells or cell lines T24 or J82 were grown to 90% confluence harvested, and counted in a hemocytometer. Total upper and lower phase GSLs were extracted and purified from equal aliquots of cells, including acetylation and deacetylation steps. GSLs were separated on HPTLC plates, then overlaid with metabolically [35 S]methionine-labeled *E. coli* R45, a wild type UTI isolated expressing Class II *pap*-encoded adhesin. Autoradiographs are shown.

A. GSLs extracted from primary cultured bladder epithelial cells and GSL standards.

Lane 1: GSL standard ceramide trihexaosyl (CTH; globotriaosyl ceramide; Gb3) from human erythrocytes; **Lane 2:** GSL standard galactosyl globoside (GG) from human kidney; **Lane 3:** sialosyl galactosyl globoside (SGG) from human kidney; **Lane 4:** lower phase GSLs extracted from primary bladder epithelial cells; **Lane 5:** upper phase GSLs extracted from primary bladder epithelial cells.

B. GSLs extracted from cultures of malignant bladder cells lines T24 and J82

Lane 1: ceramide monohexosyl (CMH; negative control) standard; **Lane 2:** CTH standard from human erythrocytes; **Lane 3:** upper phase GSLs from T24 cells; **Lane 4:** lower phase GSLs from T24 cells; **Lane 5:** GG standard from human kidney; **Lane 6:** SGG standard from human kidney; **Lane 7:** upper phase GSLs from J82 cells; **Lane 8:** lower phase GSLs from J82 cells.



Figure 4. Binding of *E. coli* R45 to glycosphingolipids partially purified from human primary vaginal epithelial cultures

Primary vaginal epithelial cells were grown to 90% confluence harvested, and counted in a hemocytometer. Total organic extraction in isopropanol:hexane:water 55:25:20 vol:vol was performed, the Folch partition was performed and the relatively crude GSLs were separated on HPTLC plates. Plates were then overlaid with metabolically [35 S]methionine-labeled *E. coli* R45, a wild type UTI isolated expressing Class II *pap*-encoded adhesin. Autoradiograph is shown. The perturbations in bands seen in this autoradiograph (e.g. lanes 5-7) as compared with other figures is caused by phospholipid contamination and co-migration with GSLs in these crude fractions.

Lane 1: standard SGG ; **Lane 2:** cervical upper phase GSLs ; **Lane 3:** cervical lower phase GSLs ; **Lane 4:** vaginal upper phase GSLs ; **Lane 5:** vaginal lower phase GSLs; **Lane 6:** primary bladder epithelial cell upper phase GSLs; **Lane 7:** primary bladder epithelial cell lower phase GSLs; **Lane 8:** primary bladder epithelial cells total GSL extract.

These data definitively demonstrate the presence of globoseries GSLs in extracts of GSLs from primary cultures of bladder epithelial cells and from primary vaginal epithelial cells. This is determined by the co-migration of specific *E. coli*-binding GSL bands with standard ceramide trihexaosyl (CTH; globotriaosyl ceramide; Gb3) from human erythrocytes, standard galactosyl globoside (GG) from human kidney, and standard sialosyl galactosyl globoside (SGG) from human kidney. As noted above, we have isolated considerably larger quantities of GSLs from the primary bladder epithelial cells than is chromatographed on this plate. The amount of GSL per lane shown in this and similar figures represents about 1/1,000 of the total amount isolated by volume. At this point in the purification of GSLs from vaginal epithelial cells, the amount of material available is comparable, although there may be some loss in subsequent steps. Thus, our data at the end of the first year of funding confirm our preliminary data suggesting that globoseries GSLs are expressed in these tissues. In addition, we have shown that we are able to isolate GSLs from these normal cell lines in adequate quantities for further characterization, such as carbohydrate structural determination. Only bands consistent with globoseries GSLs of likely known structure have been found to date, but further purification steps and HPTLC performed in other solvent systems we will use in the next year may reveal new structures.

Our data comparing GSLs from primary bladder epithelial cells with those from J82 or T24 cells show that the normal cells express larger amounts of extended globoseries GSLs. In Figure 3B, it is clear that the chemical amounts of material co-migrating with SGG in these malignant cell extracts is scant as compared with that in the normal cell extracts. These data

are consistent with glycosylation changes seen in other families of GSLs in other malignant cells, part of a large body of data generated in Dr. Hakomori's laboratory (10). The findings also support our use of normal cells, which require a more labor intensive method of cell culture than do immortalized cells. This is particularly important because of the apparent importance of SGG as a binding ligand for pap-encoded adhesin expressing E. coli (21).

As noted above, in the first year of funding, for the bacterial overlay assays with E. coli, we have primarily used metabolically labeled wild type E. coli R45 (3), which has the pap class II adhesin genotype and phenotype. As planned, the non-GSL binding laboratory strain HB101 has served as a control. Our use of the Class II-expressing organism has partially been for reasons of convenience but also because in the past year, results of epidemiological studies by our group have shown that strains bearing Class I pap-encoded adhesins (papG_{J96}) alone are absent from E. coli collected from episodes of cystitis or pyelonephritis in women, prostatitis or cystitis in men, or from groups of normal fecal isolates from men or women without UTI (22). In contrast, isolates bearing Class II or Class III adhesins are both represented among the strains from women. Thus, we have not pursued Class I-mediated binding assays.

Bacterial overlay assays have also been performed with S. saprophyticus, using crude GSL fractions from bladder and vaginal epithelial cells. Our preliminary data have been confirmed but there was the appearance of interference with binding because of the presence of contaminating and co-purifying phospholipids (to be later removed by acetylation). As shown in Figure 4, GSL extracts from primary vaginal epithelial cells appear to contain large quantities of phospholipids and these may need more purification than extracts from primary bladder epithelial cells. Thus, we postponed further binding assays using S. saprophyticus until more pure fractions were available. Since we have recently obtained these, we will be able to repeat the assays with S. saprophyticus soon.

3. Recommendations in relation to the Statement of Work

1. Our overall progress with the assays using E. coli has been as planned. We will continue to focus our assays on organisms expressing a Class II or Class III adhesin for the next funding period. Through the PCR project, we have characterized all of the E. coli isolates in our collections and now have available additional wild type organisms whose pap-encoded adhesin characteristics and adherence properties to primary bladder epithelial cells (see below) are known. We will perform initial assays to assess Class III-mediated binding pJFK102 (expressing prs-G_{J96}), but we may use a suitable wild-type organism later if this proves more practical and we cannot discern differences in binding between the cloned and wild type isolates.

2. We will repeat binding assays with S. saprophyticus, using more purified GSL samples, particularly with respect to the fractions from primary vaginal epithelial cells.

3. We will continue to use data obtained with these assays to choose bacterial-binding GSLs to purify and structurally characterize.

d. Task 4, Months 7 to 12: Immunostaining assays

1. Experimental methods, assumptions and procedure

The purpose of immunostaining assays is to confirm the identities of GSLs identified through TLC mobilities, using specific MAbs directed against the predicted GSLs. Before Dr.

Stroud left the Biomembrane Institute, we performed assays using MAbs RM-1 and ID-4 (both specific for SGG) (23). These data demonstrated positive staining of GSL bands co-migrating with standard SGG found from several different cultures of primary bladder epithelial cells (data not shown). This assay is performed according to the procedure of Magnani (24), as modified by Kannagi (25). Briefly, GSLs isolated and purified from the primary cell lines and then separated on HPTLC. After HPTLC, the plates were blocked for 2 hours in 5% BSA in PBS, washed, and incubated with appropriate dilutions of primary MAb in PBS. After an incubation with the secondary antibody, plates were washed, incubated with [125 I]-labeled protein A solution, washed, dried, and subjected to autoradiography.

Although the University of Washington has available core facilities for the use of 125 I, Dr. Stroud had already begun investigating non-radioactive methods of detecting GSL-MAb interaction on HPTLC plates. We found the potential increased safety and convenience of such methods appealing. Thus, we elected to continue developing these methods and we have achieved partial success. We have used two methods of detection of MAb binding to GSLs:

(1) creation of a silica grid for incubation of each GSL spot with its MAb, followed by overlay with alkaline phosphatase-Protein A, or by biotinylated anti-mouse with streptavidin with peroxidase and 3,3'-diaminobenzidine (DAB) as a detection reagent. There have been several problems which we have investigated in order to optimize this assay, including: (a) various blocking methods, using BSA or milk; (b) methods to keep the overlay solutions on the individual grids; and (c) methods of keeping the plate humid during incubation. As a matter of ordinary development of such an assay, we have explored various amounts of MAb added as well as various incubation times.

(2) transfer of GSLs to nitrocellulose paper. We have explored the best solvent system(s) for recovering the GSLs as well as incubation times.

2. Results and discussion

We have used standard immunostaining assays to identify SGG in GSL extracted from primary bladder epithelial cells. We have explored two non-radioactive methods of detecting GSLs in immunostaining. Although we have successfully detected GSL standards using specific MAbs in both non-radioactive methods, further development of this method is needed. This should help us to achieve greater reproducibility and convenience. If this does not work, we will return to the radioactive method.

3. Recommendations in relation to the Statement of Work

We will work 2 or 3 months more on a non-radioactive method for immunostaining. If these efforts are not successful, we will abandon this approach in favor of the traditional one.

e. Task 5, Months 13 to 24: carbohydrate structural analysis

1. Experimental methods, assumptions and procedure

At the time that this funding period began, we had purified adequate quantities of SGG from human kidney to begin structural analysis. We elected to begin our collaboration with Dr. Levery using this sample because SGG had never been structurally characterized from a normal human tissue. To characterize SGG from normal human kidney, Dr. Levery used the CCRC's techniques and equipment, including state of the art 500 MHz proton nuclear magnetic resonance (1 H-NMR) spectroscopy. Following NMR, samples were permethylated

(26) and analyzed by electrospray ionization mass spectroscopic techniques. The remaining sample has been subjected to hydrolysis, reduction, acetylation and analyzed by gas chromatography/mass spectrometry (27, 28) and we expect to receive these data in the next few weeks.

2. Results and discussion

Figure 5 shows the proton chemical shifts and $^3J_{1,2}$ coupling constants (Hz) for SGG, as well as its chemical structure. This represents the first time that SGG has been isolated and characterized definitively from a normal human tissue. We are using this standard SGG sample for the studies described herein. We have also shown that we can productively collaborate with the CCRC, an internationally recognized center for the study of complex carbohydrates. These data are presently being prepared as a manuscript to be submitted in the near future.

Table 1. Proton chemical shifts (ppm from tetramethylsilane) and $^3J_{1,2}$ coupling constants (Hz) for Sialosylgalactosylgloboside in dimethylsulfoxide-d₆/2% D₂O at 308°K.

| | NeuAc α 2 \rightarrow 3Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 3Gal α 1 \rightarrow 4Gal β 1 \rightarrow 4Glc β 1 \rightarrow 1Cer | | | | | | |
|-----------------|--|-------|-------|------------------------|-------|------------------------|-----------|
| | A | V | IV | III | II | I | R |
| H-1 | | 4.234 | 4.579 | 4.815 | 4.256 | 4.185 | 3.447 (a) |
| ($^3J_{1,2}$) | | (7.9) | (8.3) | (4.2) | (7.5) | (7.7) | 3.962 (b) |
| H-2 | | 3.279 | 3.858 | 3.766 | 3.310 | 3.040 | 3.781 |
| H-3 | 2.766 (eq) 1.346 (ax) | 3.926 | 3.675 | 3.609 | 3.399 | | 3.880 |
| H-4 | 3.538 | 3.706 | 3.880 | 3.964 | 3.815 | | 5.354 |
| H-5 | | | | 4.117 | | 3.290 | 5.538 |
| H-6 | | | | 3.476 (a) 3.454 (b) | | 3.611 (a) 3.747 (b) | 1.935 |
| Nac | 1.889 | | 1.795 | | | | |

Figure 5. Proton chemical shifts (ppm from tetramethylsilane) and $^3J_{1,2}$ coupling constants (Hz) for sialosyl galactosyl globoside in dimethylsulfoxide-d₆/2% D₂O at 308°K

3. Recommendations in relation to the Statement of Work

We plan to continue this fruitful collaboration with Dr. Levery and plan next to send him a sample of SGG purified from human primary bladder epithelial cells. We will initially focus on SGG for structural characterization and subsequent mimetic targeting because of its apparent higher avidity in binding pap-encoded adhesin expressing *E. coli* (21).

2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures

1. Experimental methods, assumptions and procedure

Primary cultures of bladder and vaginal epithelial cells were maintained and utilized as described above. *E. coli* was grown overnight on sheep blood agar plates or on antibiotic-containing Luria agar plates for cloned isolates, harvested in PBS, washed and resuspended to an OD₆₀₀ of 0.5 (corresponding to 5×10^8 organisms). *S. saprophyticus* isolates will be grown overnight shaking in trypticase soy broth and prepared similarly. The organisms were resuspended in 1.0 mL of keratinocyte medium, incubated with the cells for 3 hours, washed repeatedly with PBS and fixed and stained using a commercial Giemsa stain (Baxter). Cells treated with only a change of medium and the nonadherent laboratory isolates HB101 served as controls.

2. Results and discussion

Bacterial adherence assays were initially not planned for the first year, but we performed them as part of investigations of the GSL synthesis inhibitor PDMP, described below and to confirm our preliminary results. Since we are preparing a manuscript describing this technique, we repeated the assays as a matter of completeness. No differences have been seen with new data as compared with our preliminary results. Thus, we show only the confirmatory data below in Figure 6.

3. Recommendations in relation to the Statement of Work

These assays are highly reproducible and will be continued as planned.

b. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays

1. Experimental methods, assumptions and procedure

To demonstrate the importance of GSLs in mediating bacterial adherence, we used an inhibitor of GSL synthesis, 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), an analog of glucosylceramide that competitively inhibits the synthesis of GSLs in living cells (29). This compound has been employed to inhibit GSL synthesis in transformed human epithelial cell lines, thus reducing the adherence of *E. coli* to these cells (30). We performed pilot experiments to concomitantly show that specific GSL synthesis was inhibited by extracting and quantifying the GSLs in treated versus untreated cultures. Cells at 50% confluence were incubated with 10 μ M PDMP in keratinocyte medium for 72 hours and then used in adherence assays at 90% confluence. The overall quantity of GSLs was evaluated by separating them on HPTLC, followed by orcinol staining and bacterial adherence assays as described above, using treated and untreated primary cultures of bladder epithelial cells.

2. Results and discussion

As shown in Figure 6, the use of PDMP nearly abolished adherence of *E. coli* R45 to primary bladder epithelial cells grown in its presence, as compared with cells grown without PDMP in parallel.



Figure 6 A, B. Adherence of *E. coli* R45 and *S. saprophyticus* ST352 to primary cultures of human bladder cells.

Cells were grown to near confluence in four-chambered slides and bacteria grown overnight, washed and resuspended in keratinocyte medium at 5×10^8 organisms/mL were allowed to adhere for three hours. After extensive washing, cells were stained with a Wright-Giemsa stain. **A:** *E. coli* R45; **B:** *E. coli* R45 with PDMP-treated cells.

To confirm that the decrease in the adherence phenotype was the result of inhibited GSL synthesis, we extracted total (crude) GSLs from equal amounts of cells grown in the presence or absence of PDMP.

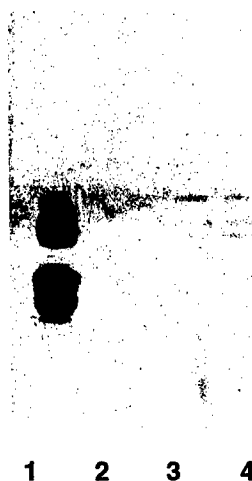


Figure 7. Binding of *E. coli* R45 to glycosphingolipids purified from human primary bladder epithelial cultures incubated with varying concentrations of PDMP, an inhibitor of GSL synthesis.

Primary bladder epithelial cells from the same original culture were grown to 90% confluence with PDMP added in varying concentrations for specific exposure times, and harvested. Cells were counted in a hemocytometer and total upper and lower phase GSLs were extracted and purified from equal aliquots of cells. GSLs were separated on HPTLC plates.

then overlaid with metabolically [^{35}S]methionine-labeled E. coli R45, a wild type UTI isolated expressing Class II pap-encoded adhesin. Autoradiograph is shown.

Lane 1: pig intestine total upper phase GSLs (positive control for globoseries GSL binding);

Lanes 2 to 5: bladder cell GSL extracts from cells grown under the following conditions:

Lane 2: 10 μM PDMP added at 10 % confluence and incubated for 72 hours; **Lane 3:** 20 μM PDMP added at 20 % confluence and incubated for 48 hours; **Lane 4:** 40 μM PDMP added at 60% confluence and incubated for 24 hours; **Lane 5:** no PDMP added; **Lane 6:** asialo GM1 GSL standard (negative control).

Many studies of the effects of PDMP have employed continuous cell lines. Thus, we have shown in a normal human primary bladder epithelial cells that PDMP causes a dose-dependent decrease in GSL synthesis. This corresponds with a near total abolition of bacterial adherence in cell cultures treated with 10 μM PDMP for 72 hours.

3. Recommendations in relation to the Statement of Work

Since these investigations were ahead of schedule and appeared successful, we plan no changes in this protocol for now.

7. CONCLUSIONS

1. We have demonstrated the feasibility of our approach in using pure, primary cultures of human bladder and vaginal epithelial cells to identify, purify and structurally characterize E. coli- and S. saprophyticus-binding glycosphingolipid moieties. We have shown that we are capable of isolating GSLs from these normal cell lines in adequate quantities for further characterization, such as carbohydrate structural determination.
2. We have established primary cultures of vaginal epithelial cells de novo in our laboratory, a technique which we expect to be useful in our further studies for years 2 to 4.
3. We have definitively demonstrated the presence of globoseries GSLs in extracts of GSLs from primary cultures of bladder epithelial cells and from primary vaginal epithelial cells, confirming our preliminary data obtained with much more crude GSL extracts. Preliminary data also confirm our findings of ganglioseries GSLs in these cell lines.
4. Only bands consistent with globoseries GSLs structures predicted to be known have been found to date, but further purification steps and HPTLC performed in other solvent systems we will use in the next year may reveal new structures.
5. We have shown that GSLs from primary bladder epithelial cells contain larger amounts of extended globoseries GSLs, in particular a structure co-migrating with standard SGG, as compared with GSLs extracted from the malignant bladder cell lines J82 or T24. The chemical amounts of material co-migrating with SGG in the cell extracts from J82 or T24 cells is scant as compared with that in the normal cell extracts. These data support our use of primary cultures of bladder and vaginal epithelial cells, which require a more labor intensive method of cell culture than do immortalized cells.
6. We have performed the first unambiguous structural determination of SGG isolated from a normal human tissue.
7. We have confirmed our preliminary finding that primary bladder epithelial cells serve as a useful model for studying the adherence of uropathogenic bacteria in the pathogenesis of UTI.
8. Our data using an inhibitor of GSL synthesis, PDMP, demonstrate the critical importance of GSLs in the adherence of uropathogenic E. coli to bladder epithelium.

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9. APPENDICES

1. National Institutes of Health biosketch form for Dr. S. Lavery

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

| | |
|--------------------------|---|
| NAME Steven B. Levery | POSITION TITLE Co-Technical Director and Assistant Research Biochemist |
|--------------------------|---|

EDUCATION (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

| INSTITUTION AND LOCATION | DEGREE | YEAR CONFERRED | FIELD OF STUDY |
|--|--------|-------------------|-------------------|
| Northeastern University, Boston, Massachusetts | B.A. | 1971 | Chemistry |
| Northeastern University, Boston, Massachusetts | M.S. | 1976 | Organic Chemistry |
| University of Washington, Seattle, Washington | Ph.D. | 1993 | Chemistry |

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Experience:

| | |
|--------------|--|
| 1967-1970 | Chemical Technician, Lever Brothers Co., Research and Development (Cooperative Education Program), Edgewater, New Jersey |
| 1972-1973 | Chemist, Collaborative Research Inc., Waltham, Massachusetts |
| 1974-1976 | Teaching Assistant, Chemistry Department, Northeastern University, Boston, Massachusetts |
| 1977-1978 | Research Technologist, Orthopedic Research, Children's Hospital Medical Center, Boston, Massachusetts |
| 1979-1980 | Research Technologist, Department of Physiology and Biophysics, University of Washington, Seattle, Washington |
| 1980-1981 | Research Technologist, Mass Spectrometry Laboratory, Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center, Seattle, Washington |
| 1981-1986 | Supervisor, Mass Spectrometry Laboratory, Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center |
| 1986-1994 | Staff Scientist and Head, Laboratory for Analytical/Structural Biochemistry, The Biomembrane Institute, Seattle, Washington |
| 1995-1996 | Senior Scientist, Protein Analysis, Perkin Elmer-Applied Biosystems Division, Foster City, California |
| 1996-Present | Co-Technical Director, NIH Resource Center for Biomedical Complex Carbohydrates, Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia |
| 1997-Present | Assistant Research Biochemist, Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia |

Publications: (from a total of 81)

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REPLY TO
ATTENTION OF:

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21 Feb 03

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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